SUPPLEMENTAL MATERIAL

Methods

All procedures were approved by the Institutional Animal Care and Use Committee at Vanderbilt University. Given the recognition by our work and others' of a gender effect on repolarization in mice,[1,2] these experiments were conducted in myocytes from adult females. In some experiments, the effect of chronic dofetilide on action potentials and late sodium current were determined in cardiomyocytes isolated 5 days after a large intraperitoneal dose of dofetilide (30 mg/Kg).

Effect of drug exposure on Akt phosphorylation

Either HEK cells stably transfected with SCN5A (obtained from the laboratory of Al George at Vanderbilt) or CHO cells transiently transfected with SCN5A were grown to near confluency, and treated for 1 hour with LY294002 (20µM), moxifloxacin (100µM), dofetilide (3µM), or DMSO vehicle in normal culture conditions. After washing with PBS, cells were lysed using CelLytic MT (Sigma Aldrich, St. Louis, MO) supplemented with Halt protease/ phosphatase inhibitors (Promega, Madison, WI). Protein lysates (30ug per lane) were electrophoresed on 10% Nu-PAGE gel (BioRad, Hercules, CA). After transferring to nitrocellulose and blocking with Odyssey blocking buffer (Licor, Lincoln, NE), blots were incubated in primary antibodies overnight at 4°C. Blots were incubated 1 hour in secondary antibodies then imaged on Odyssey system. Antibodies used were mouse anti α-tubulin (Cell Signaling Technology, Danvers, MA) at 1:2000, rabbit anti-pAkt (S473) (Cell Signaling Technology, Danvers, MA) at 1:250, donkey anti-mouse IRDye800CW and donkey anti-rabbit IRDye680LT (LiCor, Lincoln, NE) at 1:10,000. ImageJ software was utilized for quantification of Western blot bands. pAkt signal was normalized to the α-tubulin control and reported as percentage increase/decrease over DMSO control.

Nav1.5 Western blotting

Nav1.5 channel protein abundance was assessed in the absence and presence of a 48 hour dofetilide exposure to SCN5A-transfected CHO cells, as previously described.[1] Lysates were generated by pulverizing flash frozen sections of ventricular tissue followed by homogenization in a Dounce apparatus with 1xRIPA (150 mM NaCl, 50 mM Tris, pH 7.5, 1% NP-40 (IGEPAL), 0.5% Sodium deoxycholate, and 0.1% sodium dodecyl sulphate in 1x DPBS pH7.5) buffer. The homogenates were centrifuged at 10 000g for 5min at 4°C and the supernatant lysates were transferred to new chilled micro-centrifuge tubes. Lysate protein concentration was analyzed using a bicinchoninic acid assay (Pierce Biochemicals) following the manufacturer's instructions. Twenty to 80 µg of lysate from each cardiac preparation was separated on NuPage 8% Tris-Acetate gels (Invitrogen). Separated proteins were transferred to 0.2 µm nitrocellulose membranes (Amersham Biosciences) and were blocked overnight in blocking buffer comprised of 0.05% Tween-20 Tris–buffered saline (TTBS) plus 5% non-fat dry milk at 4°C, and then were incubated with immunoglobulins against Na_V1.5 (pAb 1:200, Alomone Labs) and calnexin (pAb 1:1000, Stressgen BioReagents) at room temperature for 2 hours. Membranes were washed three times with TTBS for 10 min each and incubated with secondary anti-rabbit horseradish peroxidase-linked antibodies (Amersham Biosciences) in TTBS at room temperature for 1 h. Blots were then washed four times for 10 min each in TTBS. We visualized antibody interactions after transfer of light images to autoradiography film using the ECL system (Amersham Biosciences). Blot images were then scanned using a BioRad ImageOne processor and subjected to image densitometry using the ImageJ software (http://rsb.info.nih.gov/ij/) and averaging applications were performed in Microsoft Excel.

FuGENE6-mediated *SCN5A* channel expression and cell transfection

These methods are similar to those reported previously and are described in the on-line supplement. Recombinant cDNA (2 µg) for human *SCN5A* encoding the human cardiac sodium channel Nav1.5 was transiently transfected in Chinese hamster ovary (CHO) cells, as previously reported.[3,4] In brief, *SCN5A* DNA (Gen Bank accession No. NM000335) was subcloned into the pRc-CMV vector (Stratagene) and transiently transfected into cultured CHO cells using FuGENE6 (Roche Applied Bioscience). A plasmid encoding the enhanced green fluorescent protein (pEGFP-N3, BD Bioscience Clontech) was cotransfected to identify transfected cells for electrophysiologic study. Beta-subunits were not cotransfected. Cells were studied at 48 hours after transfection with or without drug exposures as noted in Results before study.

Peak and late sodium current recordings

Whole-cell voltage clamp experiments were conducted at room temperature (22-23°C; 18°C for mouse myocytes). To record sodium currents, two extracellular bath solutions were used. In CHO cells and hiPSC-CMs, the external solution contained (in mmol/L) NaCl 135, KCl 4.0, $MgCl_2$ 1.0, $CaCl_2$ 1.8, glucose 10, and HEPES 10; the pH was 7.4, adjusted with NaOH. In mouse ventricular myocytes, the external solution was K^+-free and Ca^{2+}-free with a lower sodium concentration (20 mmol/L) to provide voltage control. In some mouse myocyte experiments, we used an external K^+-free and Ca^{2+}-free solution with a sodium concentration of 135 mmol/L to optimize I_{Na-L} recording, as previously reported.[5,6] The pipette (intracellular) solution contained (in mmol/L) NaF 5, CsF 110, CsCl 20, EGTA 10, and HEPES 10; the pH was 7.4, adjusted with CsOH. To eliminate L- and T-type inward calcium currents as well as outward potassium currents in mouse cardiomyocytes and hiPSC-CMs, 1 μmol/L nisoldipine, 200 μmol/L $NiCl_2$, and 500 μmol/L 4-aminopyride (4-AP) were added into the bath solution, respectively. In some experiments (as noted in Results), PIP3 (1 μM) was added to the pipette solution. To record potassium currents in mouse ventricular myocytes, the pipette (intracellular) solution contained (in mmol/L) KCl 110, K_4BAPTA 5, K_2ATP 5, $MgCl_2$ 1, and HEPES 10, with pH adjusted to 7.2; the extracellular solution was normal Tyrode's containing (in mmol/L) NaCl 135, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10, and glucose 10, with pH adjusted to 7.4.

Glass microelectrodes were heat polished to tip resistances of 0.5-2 MΩ. Cells were held at -120 mV, and sodium current was elicited with a single 200-ms pulse from -120 to -30 mV at which maximal peak inward sodium current is usually observed. Data acquisition was carried out using an Axopatch 200B patch-clamp amplifier and pCLAMP version 9.2 software (MDS Inc., Mississauga, Ontario, Canada**)**. Currents were filtered at 5 kHz (−3 dB, four-pole Bessel filter) and digitized using an analog-to-digital interface (DigiData 1322A, MDS Inc.). To minimize capacitive transients, capacitance and series resistance were corrected ~80%. Current magnitudes were normalized to cell size, generated from the cell capacitance calculated by Membrane Test (OUT 0) in pClamp 9.2. Clamp protocols used are shown on the figures. I_{Na-L} was averaged in a 3 ms time window (195-198 ms after the pulse) before the capacity transient at the end of a 200 ms depolarizing pulse. Electrophysiological data were analyzed using pCLAMP version 9.2 software and the figures were prepared by using Origin 8.5.1 software (OriginLab Corp., Northampton, MA, USA).

Action potential recordings

In current-clamp mode, APs from isolated mouse ventricular myocytes were elicited by injection of a brief stimulus current (1–2 nA, 2–6 ms, variable stimulation frequencies). APs in human iPSC-CMs were recorded from spontaneously-beating clusters at Day 30-35 post-induction. For AP experiments, the bath (extracellular) solution contained (in mmol/L): NaCl 135, KCl 4.0 , $CaCl_2$ 1.8, and $MgCl_2$ 1, HEPES 5, glucose 10, with a pH of 7.4 (adjusted by NaOH). The pipette-filling (intracellular) solution contained (in mmol/L): 120 Aspirate-K, 25 KCl, 4 ATP-Na_2, 1 $MgCl_{2,}$ 2 Phosphocreatine-$Na_{2,}$ 2 GTP-Na, 1 $CaCl_{2,}$ 10 EGTA, and 5 HEPES, with a pH of 7.3 (adjusted by KOH). Microelectrodes with tip resistances of 3-5 mΩ were used. Ten successive traces were averaged for analysis of action potential durations at 50 and 90% repolarization (APD_{50} and APD_{90}). Action potentials were recorded prior to and after exposure to drug for variable times as noted in Results.

References

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Supplemental Figures

Supplemental Figure 1

Representative current traces show the late current changes caused by chronic exposure of *SCN5A*-transfected CHO cells to verapamil, erythromycin and thioridazine.

Supplemental Figure 2

The selective late current blocker ranolazine inhibited late current increased by chronic exposure of *SCN5A*-transfected CHO cells to three potent I_{Kr} blocking drugs (dofetilide, d-sotalol and E-4031). Ranolazine had only minor effects on peak current at this concentration.

Supplemental Figure 3

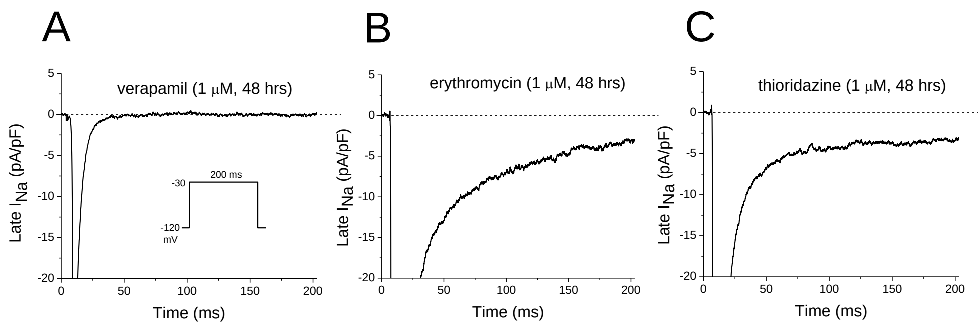
Late current recorded at an external sodium concentration ($[Na^+]_o$) of 135 mM was increased at Day 5 after intraperitoneal (i.p.) injection of a single dose of dofetilide (30 mg/Kg) in female adult mice, consistent with the effect of a 5 hour exposure to dofetilide in mouse cardiomyocytes. These recordings were performed with an extracellular sodium of 135 mM.

Supplemental Figure 4

These tracings show examples of current recorded 48 hours after SCN5A transfection in the absence (A) or presence (B) of dofetilide in the medium, and summary current-voltage data (panel C). The increased peak current with dofetilide was also seen with d-sotalol and E-4031 (panel D). Panel E shows that Western blots did not reveal any difference in total Nav1.5 protein abundance in *SCN5A*-transfected CHO cells grown for 48 hours with chronic dofetilide (1 µM, n=6 each).

Supplemental Figure 5

Effects of 48 hours dofetilide exposure in CHO cells on the time course of sodium current inactivation and recovery from inactivation. **A-C**: Traces recorded using the protocol indicated in control cells (**A**) and cells exposed for 48 hours to dofetilide (**B**) show faster recovery in the dofetilide-exposed cells; summary data are shown in panel **C**. **D.** Chronic dofetilide also increased both the fast and slow time constants of sodium current inactivation.



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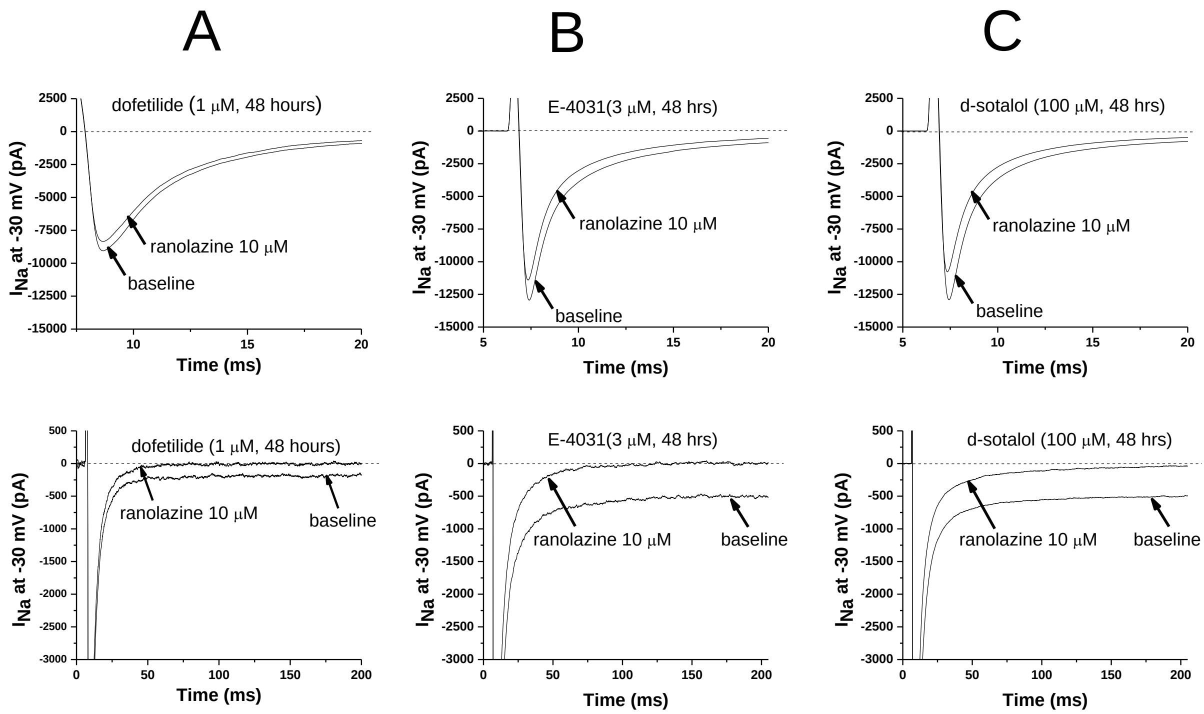

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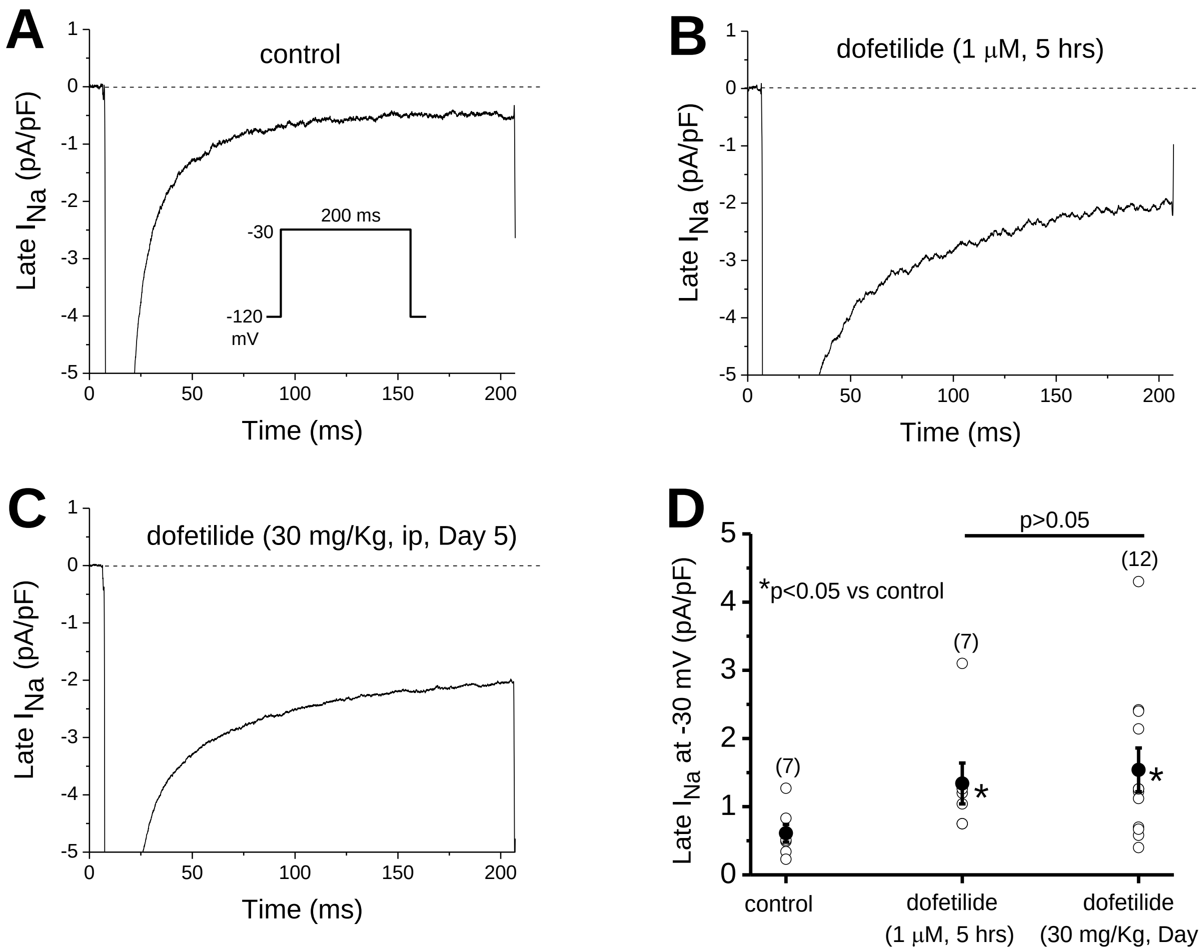

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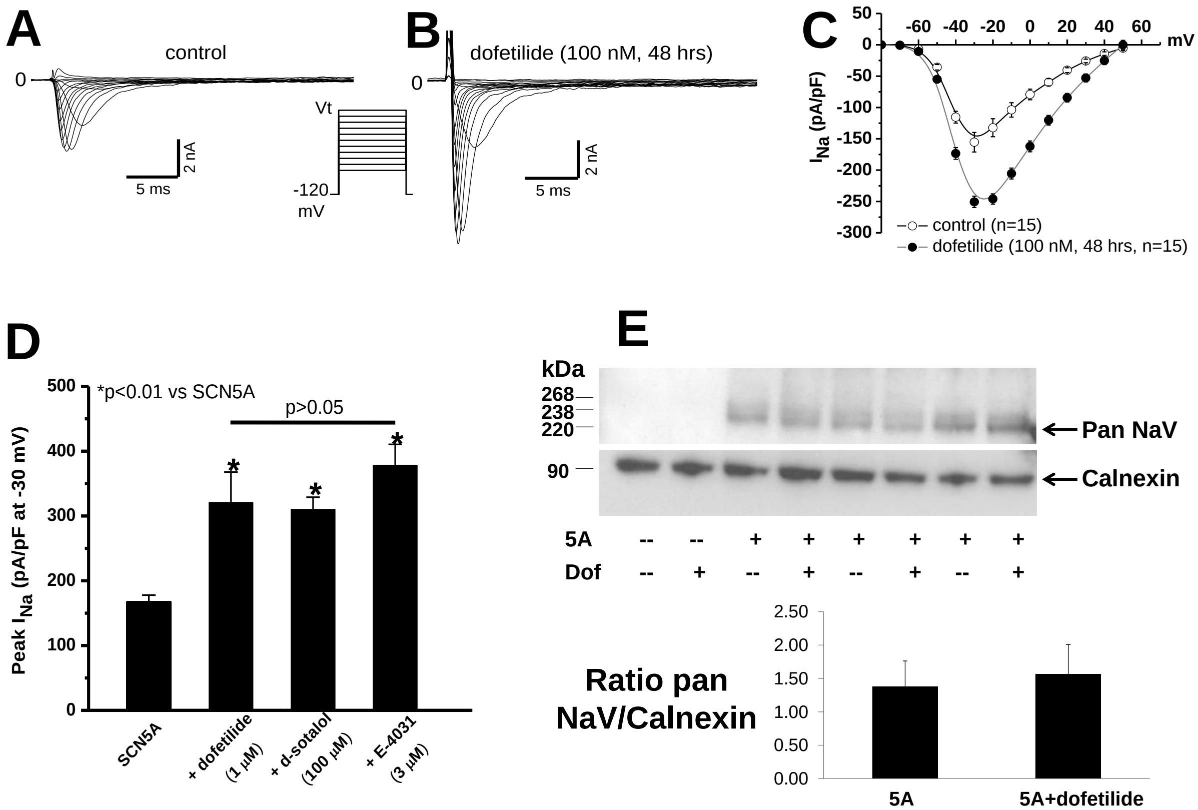

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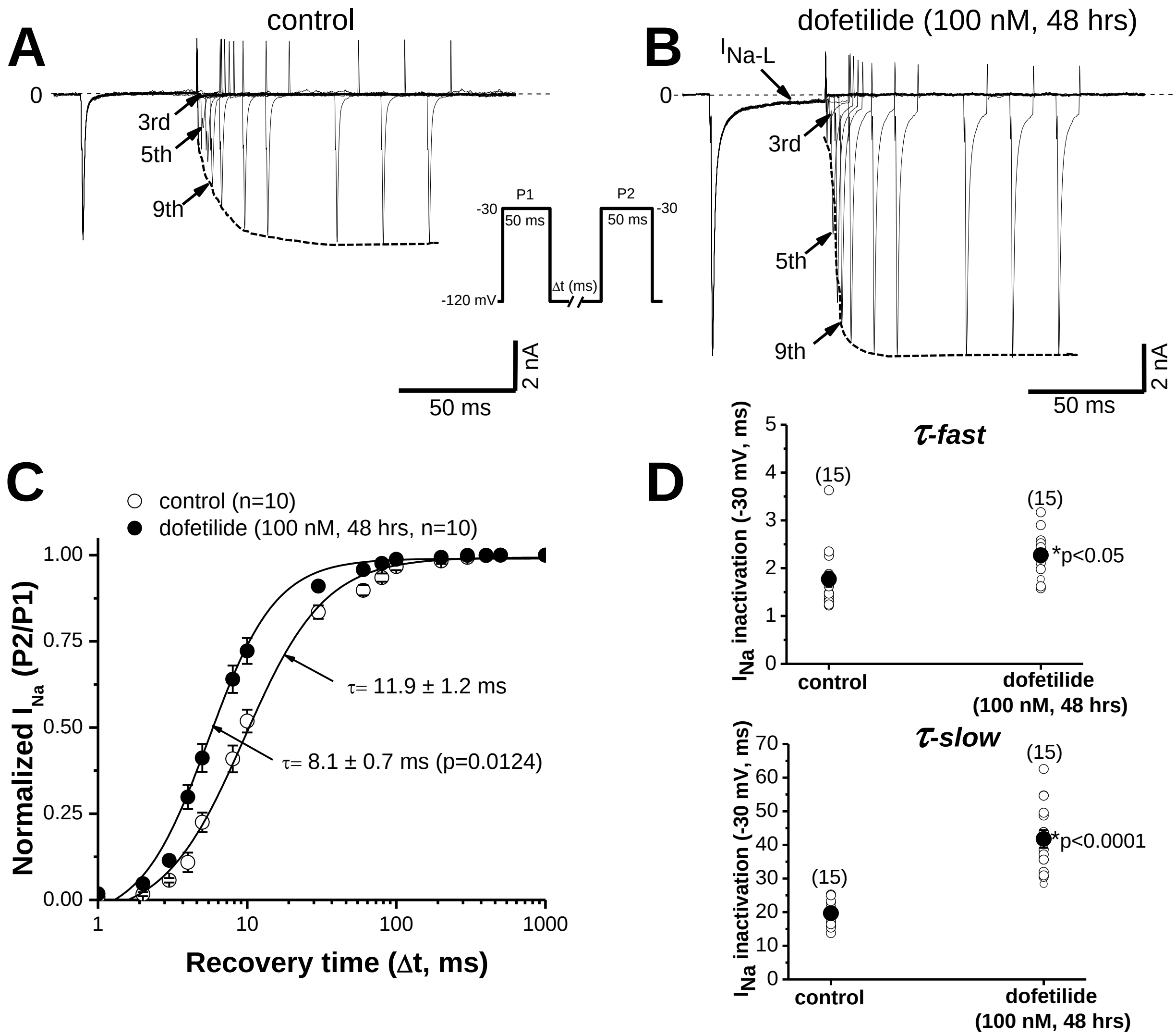

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